Spore Maturation and Release of Two Evergreen Macaronesian Ferns, *Culcita macrocarpa* and *Woodwardia radicans*, along an Altitudinal Gradient

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Abstract.—The variables affecting spore phenology have been poorly studied in contrast with the abundant literature on leaf phenology. This paper deals with the influence of altitude and canopy cover on spore maturation and release of *Culcita macrocarpa* and *Woodwardia radicans* in the island of São Miguel, Azores. The study was conducted during one sporing season at three altitudes (400, 600, and 800 m). In both species spore maturation occurred in autumn and may be controlled by the previous accumulation of photosynthates. Spores were not released until late winter owing to a requirement for dry weather conditions. Dispersal took place later at higher altitude, due to lower temperature and higher humidity. This gradual liberation of spores along an altitudinal gradient is important for the endemic Azores bullfinch *Pyrrhula murina* (a bird that feeds on spores in winter), providing food over an extended period.

Key Words.—Azores, altitudinal gradient, spore phenology, laurel forest, *Culcita*, *Woodwardia*, *Pyrrhula*, Blechnaceae, Culcitaceae

Diaspore (seed or spore) maturation and dispersal play a key role in plant population dynamics. Diaspore dispersal is a prerequisite for the establishment of new populations and a vehicle for gene flow between populations (Haufler, 2002). The selective forces that influence the timing of flowering, fruiting and seed dissemination have been widely investigated (Fenner, 1998). In contrast, few studies have dealt with fern spore maturation and dispersal (e.g., von Aderkas and Green, 1986; Durand and Goldstein, 2001; Sawamura *et al.*, 2009). Spore production is affected by several environmental factors, such as temperature, humidity and canopy cover (Odland, 1998; Greer and McCarthy, 2000; Arens, 2001).

Altitudinal gradients are powerful 'natural experiments' for testing ecological and evolutionary responses of organisms to abiotic factors. There are two categories of environmental changes with altitude: those physically tied to meters above sea level, such as temperature; and those that are not generally

altitude specific, such as hours of sunshine (Körner, 2007). Altitudinal ecological gradients reflect differences in genetic (Herrera and Bazaga, 2008), vegetative (Scheidel and Bruelheide, 2004), reproductive (Dangasuk and Panetsos, 2004) and phenological traits (Schuster *et al.*, 1989). In some fern species, spore production decreases toward higher and lower altitudinal distribution limits (Sato *et al.*, 1989; Odland, 1998).

We studied the influence of altitude on spore maturation and dispersal of *Culcita macrocarpa* C. Presl. (Culcitaceae) and *Woodwardia radicans* (L.) Sm. (Blechnaceae). These ferns occur in a warm-temperate range that extends discontinuously through Macaronesia (Azores, Madeira and Canary islands), the Atlantic coast of the Iberian Peninsula and, in the case of *W. radicans*, some locations in the Mediterranean region. Both species are considered relicts of the tropical flora that covered the Mediterranean area during the Tertiary period (Pichi-Sermolli, 1979). *Culcita macrocarpa* and *W. radicans* share the same life-form, with large shoots that grow above ground and evergreen leaves over two meters long, which makes them the largest ferns in Europe.

Culcita macrocarpa and *W. radicans* abound in the Azores (Dias, 1996), the wettest and northernmost Macaronesian archipelago, where they occur along a large altitudinal gradient of 300–1000 m for *C. macrocarpa* and 50–950 m for *W. radicans* (Schäfer, 2002). This makes the Azorean populations a suitable model to study the effects of altitude-correlated environmental factors on spore maturation and release. Additionally the sporangia of these two species are important winter food resources (Ramos, 1995, 1996a) for the critically endangered Azores bullfinch (*Pyrrhula murina* Godman). This bird is restricted to about 6000 ha in the east of the island of São Miguel, from which only 1675 ha correspond to native forest, largely invaded by exotic species (Ramos, 1996b; Ceia, 2008). We compared the spore phenology traits of *C. macrocarpa* and *W. radicans* at three altitudes in São Miguel island. Our specific questions were: (1) What are the effects of altitude on temperature, humidity and vegetation cover? (2) Is the timing and success of spore maturation influenced by temperature, humidity and vegetation cover? (3) Does the altitudinal gradient affect the dates of spore release? (4) What are the implications for the conservation of the Azores bullfinch?

MATERIALS AND METHODS

Study sites.—The study was conducted in Serra da Tronqueira, São Miguel Island, archipelago of the Azores (37°47′N, 25°13′W). This area is a steep volcanic range with oceanic climate (Marques *et al.*, 2008). Temperatures are mild throughout the year (mean annual temperature 17°C at sea level) and there is no frost. Yearly rainfall increases with altitude varying from ~1500 mm at sea level to >3000 mm at highest altitudes (~1100 m). The canopy of the natural laurel forest is dominated by evergreen trees and shrubs [*Erica azorica* Hochst. ex Seub., *Frangula azorica* V. Grubov, *Ilex perado* Aiton ssp. *azorica* (Loes.) Tutin, *Juniperus brevifolia* (Seub.) Antoine, *Laurus azorica* (Seub.)

Franco, *Myrsine africana* L., *Prunus lusitanica* L. ssp. *azorica* (Mouillef.) Franco, *Vaccinium cylindraceum* Sm. and *Viburnum tinus* L. ssp. *subcordatum* (Trel.) P. Silva]. Most of the original forest has been converted to plantations of *Cryptomeria japonica* (L. fil.) D. Don (300–900 m) or invaded by alien species: *Hedychium gardnerarum* Sheppard ex Ker-Gwal. (0–950 m), *Clethra arborea* Aiton (500–900 m) and *Pittosporum undulatum* Vent. (50–650 m) (Schäfer, 2002). Ferns are rare to absent in patches of dense homogenous exotic vegetation.

To study environmental and spore phenology variables (maturation and release), we selected three sites with both *C. macrocarpa* and *W. radicans* at 400, 600 and 800 m (hereafter referred to as low, mid and high altitude, respectively). The low altitude site was a laurel forest densely invaded with *P. undulatum* and *Acacia melanoxylon* R. Br., whereas, the mid altitude site was a laurel forest moderately invaded by *C. arborea*. The high altitude site was a laurel forest mixed with *Pinus nigra* Arn. and *C. japonica* plantations. At each altitude 12 mature individuals (i.e., with at least one fertile leaf) of each species were randomly selected and tagged, yielding a total of 72 marked individuals (12 individuals × 3 altitudes × 2 species).

Environmental variables.—Temperature and relative humidity measures were obtained with a thermohigrometer (HOBO Pro v2 logger, Onset Computer Corporation, USA) at each altitude (400, 600 and 800 m). These thermohigrometers were placed 1.5 m above the ground under tree canopy. Data were hourly recorded for one year starting in April 2007. To determine canopy cover, hemispherical photographs at 1.3 m over each tagged individual fern were taken using a digital camera (Nikon CoolPix 995, Nikon, Japan) with a fish eye converter (FC-E8, Nikon, Japan). Photos were orientated to the magnetic north and horizontally located using a bubble level (Valladares, 2006). Images were processed with Gap Light Analizer 2.0 (Forest renewal BC, Canada). Canopy cover (%) was calculated as 100 − canopy openness (%), the later being percentage of open sky seen from beneath a forest canopy.

Spore phenology variables.—From 30 October 2006 to 15 May 2007, the six study populations were visited every ca. 10 days to assess whether timing of spore maturation and release differed with altitude. At the base of a fertile pinna of each tagged individual, two opposite pinnules were marked, one to study spore maturation and the other to study spore release. Maturation was studied by collecting six sori per pinnule in each visit until the beginning of spore release (9 February 2007). Sori were stored in Eppendorf vials to keep sporangia hydrated and avoid spore release. In the laboratory, sporangia were opened with a lancet and their content was observed with a light microscope. A random sample of 400 spores per individual were sorted into three morphological groups: mature, immature and aborted. Mature spores have a two-layered wall, with both perispore and exospore, and their protoplast is fulfilled with lipid drops (Tryon and Lugardon, 1991). Immature spores lack a perispore and oil drops and aborted spores lack a protoplast and/or are collapsed. Spore maturation date was defined as the number of days since January 1 (i.e., Julian days) until an individual possessed 90% mature spores.

The percent of aborted spores was determined from the spore sample of the visit preceding the spore release date of each individual (see below).

Indusia opening was used to estimate the timing of spore release. Both studied species have indusia that completely enclose the sori and as soon as indusia open, most spores are released out of the sori (pers. observation). During each visit we counted on the marked pinnules the number of sori with open indusia. Spore release date was defined as the number of Julian days until 50% of an individual's indusia were open.

Statistical analyses.—Generalized Linear Models (GLMs; McCullagh and Nelder, 1989) were built for the following variables: canopy cover percentage, spore maturation date, spore release date and spore abortion percentage using the GENMOD procedure of SAS 9.0 (SAS Institute, 2002). GLMs were used because these variables departed from the normal distribution. A binomial distribution with logit link function was used for canopy cover percentage and abortion percentage, and a Poisson distribution with log link for maturation date and release date because under these conditions the explained variation was maximal. The explanatory variables considered in the models were fern species (*C. macrocarpa* and *W. radicans*) and altitude (low, mid and high); canopy cover percentage was included as a covariate in the maturation date and release date models. All of these variables were considered as fixed effects. Subsequent pairwise comparisons were made using LSMEANS statement of SAS 9.0 (SAS Institute, 2002). The relationship between maturation date and release date was assessed by Spearman's rank correlation coefficient within each species. This analysis was performed with SPSS 13.0 (SPSS, 2003). Data are shown as mean ± SE unless otherwise specified.

RESULTS

Environmental variables.—Temperature decreased with increasing altitude. Yearly means were 15.5°C, 13.6°C, and 12.4°C in the low, mid and high altitudes, respectively. The average altitudinal gradient was −0.78°C/100 m [= (12.4°C–15.5°C)/(800 m–400 m)]. Temperatures were mild throughout the year (Fig. 1A), with only 7°C difference between the warmest (July, August) and the coldest (February, March) months in the three altitudes (Fig. 1A). The winter was frost free, with absolute minimums above 4°C. Relative humidity increased with increasing altitude (Fig. 1B). In the three altitudes monthly means were generally far above 85% although humidity decreased during spring and summer, with absolute minimums below 40%. Canopy cover percentage differed significantly among altitudes but not between fern species (Table 1, Fig. 2). Cover increased in the order: mid < low < high, with 63% ± 2, 71% ± 3 and 83% ± 1, respectively (data for both fern species pooled).

Spore maturation and abortion.—At the beginning of the study (October 30) *C. macrocarpa* had greater than 70% mature spores at the three altitudes (Fig. 3). *Woodwardia radicans* showed a similar percentage at low altitude, whereas percentages were lower at mid and, especially, low altitudes. This initial difference among altitudes disappeared with time and maturation date,
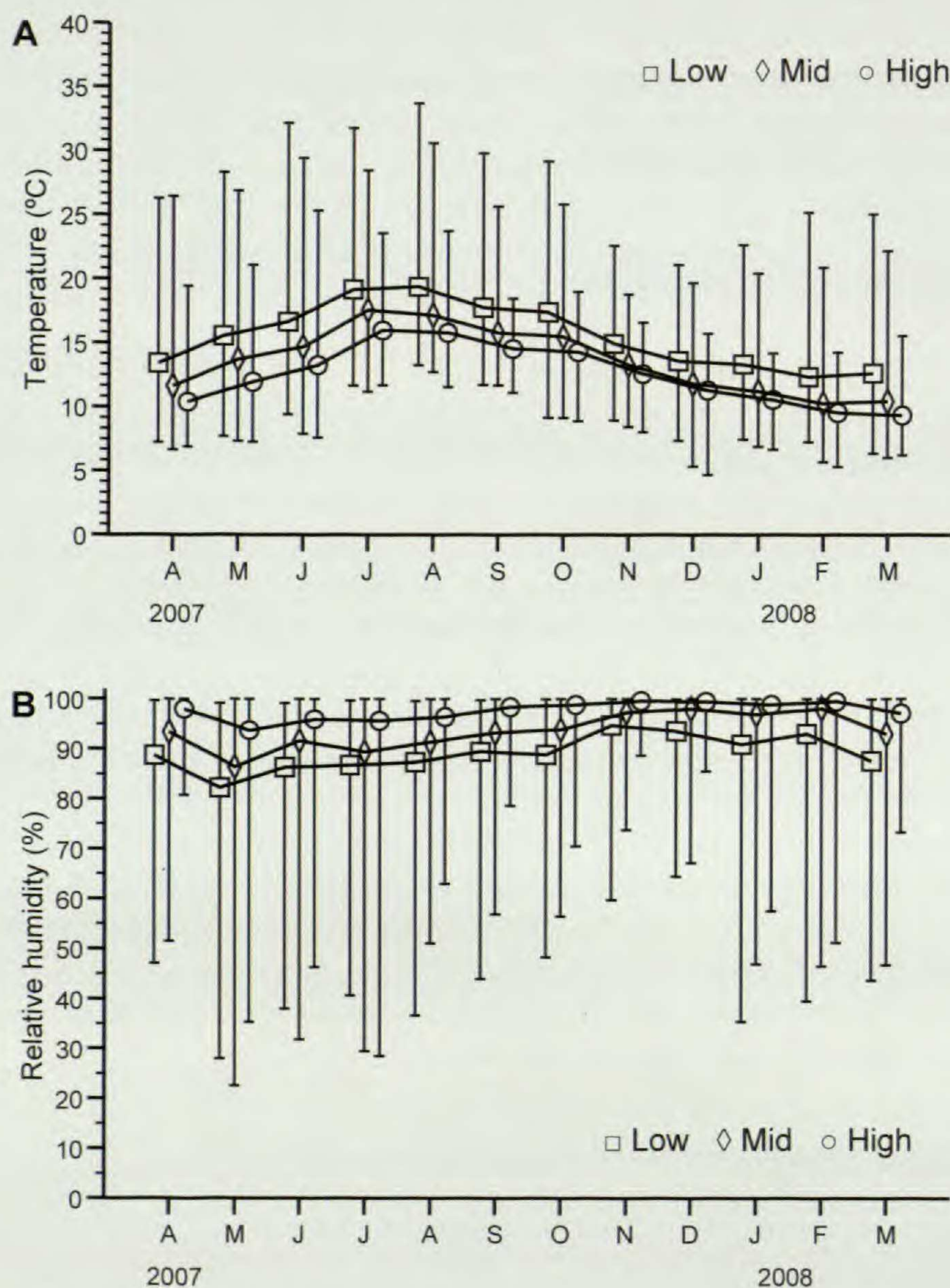
Fig. 1.   Monthly temperature (A) and relative humidity (B) at the three study altitudes (mean ± absolute maximum and minimum). Climatic data were recorded hourly with thermohigrometers placed 1.5 m above the ground under tree canopy. Bars are the highest and lowest value in each month.

i.e., the number of days before reaching 90% mature spores, was not significantly affected by altitude (Table 1). Canopy cover also did not have a significant effect on maturation date. Differences between species were significant, with earlier maturation in *C. macrocarpa* [315 Julian days (= November 11) ± 4 days, data from the three altitudes pooled] than in *W. radicans* [345 Julian days (= December 11) ± 6 days]. Neither species reached 100% mature spores at the end of the study period (Fig. 3). This was mainly due to existence of some aborted spores in all study individuals. Abortion percentage did not differ significantly between species or among sites (Table 1). Both species had low abortion percentages at the three altitudes (means ≤ 8%).

TABLE 1. Summary of GLMs for the effects of species (*Culcita macrocarpa* or *Woodwardia radicans*) and altitude (low, mid or high) on canopy cover percentage, spore maturation date, spore abortion percentage and spore release date. Canopy cover percentage was considered as a covariate in the models for maturation date and release date. Significant values are in bold. d.f. = degrees of freedom.

Variable	Effect	d.f.	χ^2	*P*
Canopy cover (%)	species	1	0.01	0.908
	altitude	2	54.02	**<0.0001**
	species × altitude	2	1.16	0.559
Maturation date	species	1	12.16	**0.0005**
	altitude	2	5.28	0.071
	canopy cover	1	0.49	0.483
	species × altitude	2	4.86	0.088
Abortion (%)	species	1	0.01	0.922
	altitude	2	5.02	0.081
	species × altitude	2	5.66	0.059
Release date	species	1	0.66	0.416
	altitude	2	6.11	**0.047**
	canopy cover	1	0.38	0.536
	species × altitude	2	0.06	0.968

Spore release.—In both species, spore release started in mid-January and ended in early May (Fig. 4). Spore release of each individual was gradual, and for *C. macrocarpa* highly synchronous among individuals at all altitudes, as revealed by the small standard errors. *Woodwardia radicans* was less synchronous at high altitude. Release date (number of days before reaching 50% indusia open) was only significantly affected by altitude (Table 1).
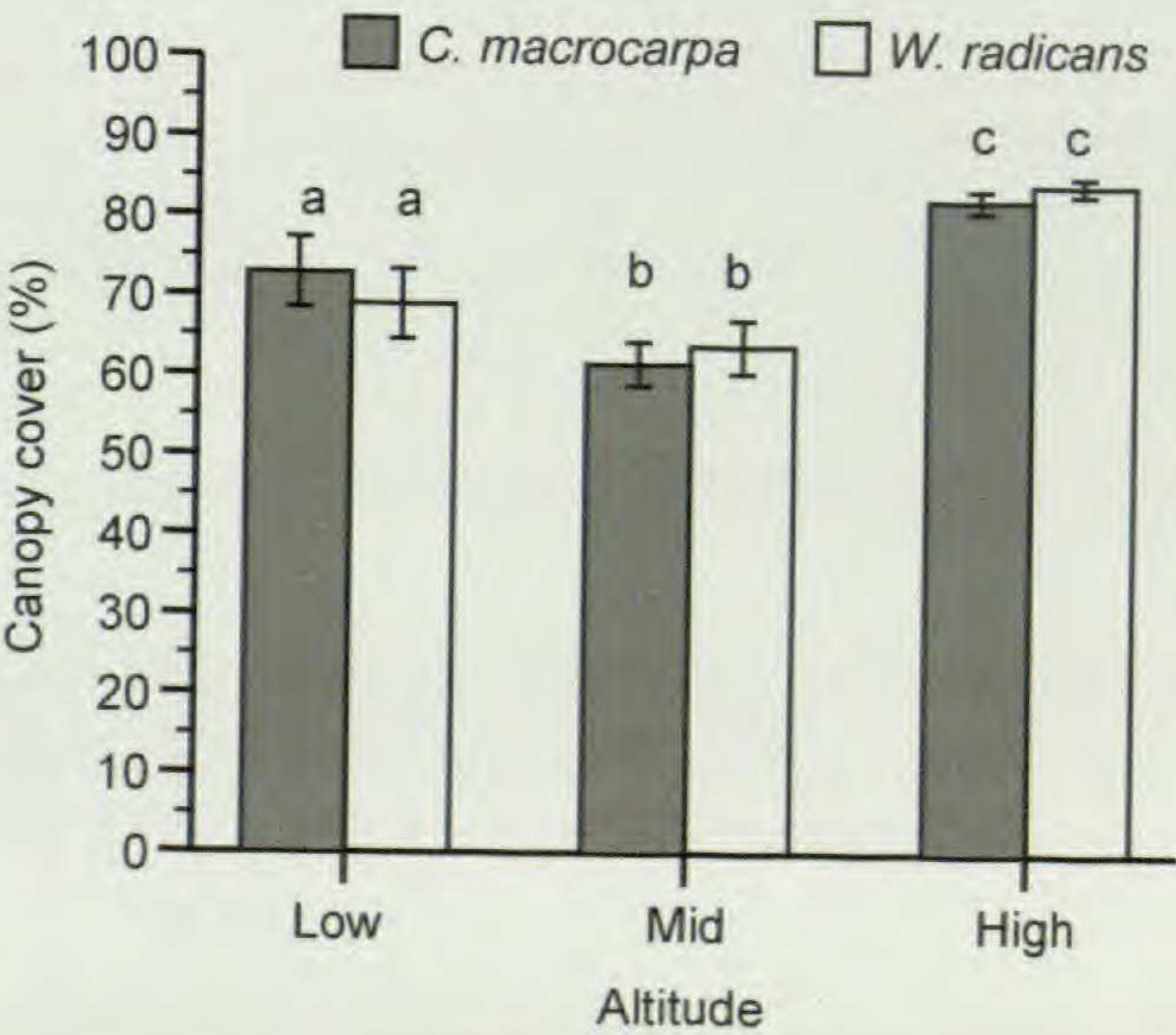

FIG. 2. Percent canopy cover (mean ± SE) for *Culcita macrocarpa* and *Woodwardia radicans* at three altitudes. Each mean represents twelve hemispherical photographs taken over each tagged individual (n = 12). Different letters indicate significantly different means ($P < 0.05$, LSMEANS; SAS Institute, 2002).
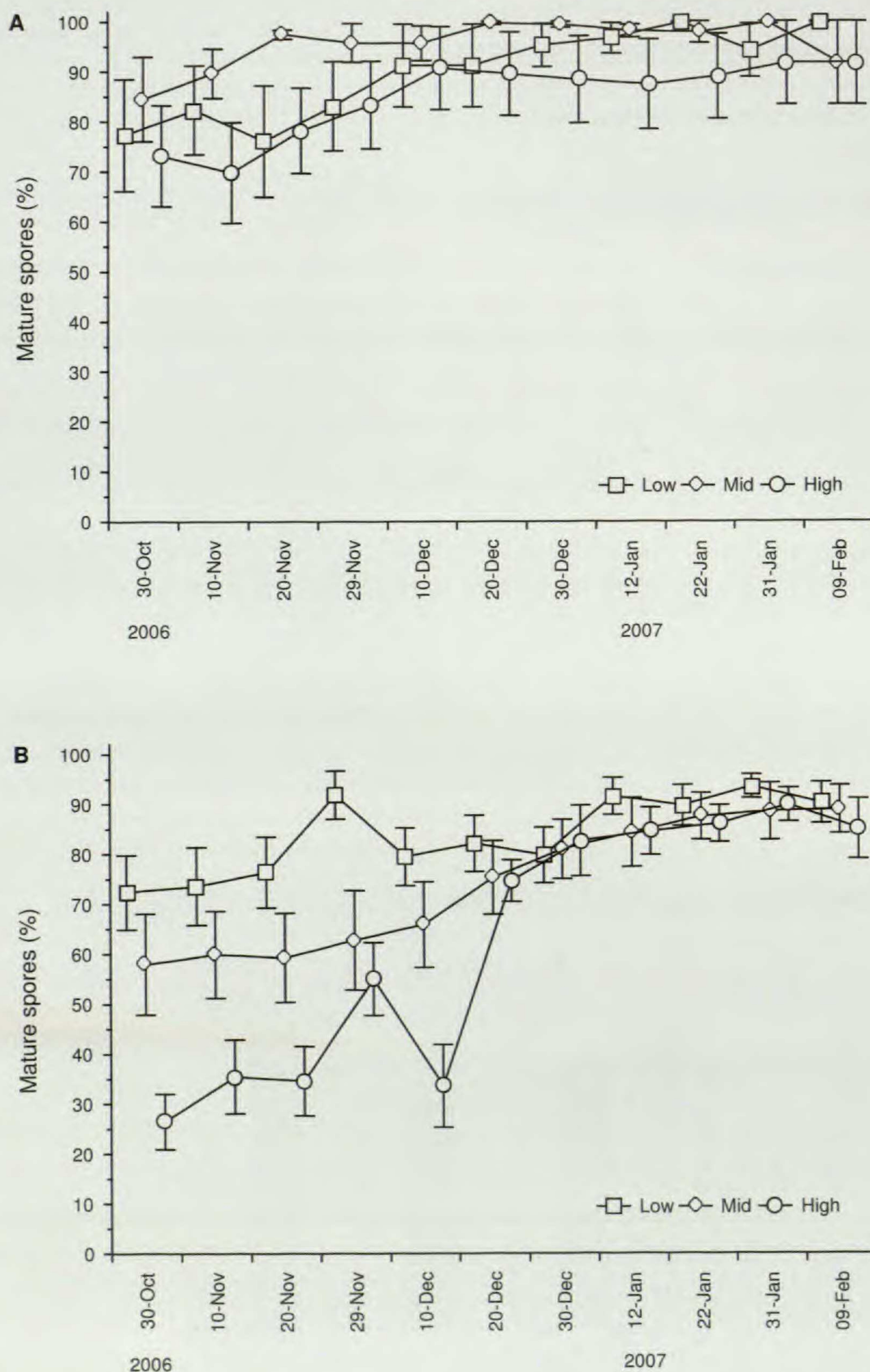
FIG. 3. Seasonal changes in spore maturation (percent mean ± SE) for *Culcita macrocarpa* (A) and *Woodwardia radicans* (B) at three altitudes. Each mean represents twelve permanently marked individuals (n = 12). Four hundred spores per individual plant were observed for each date.
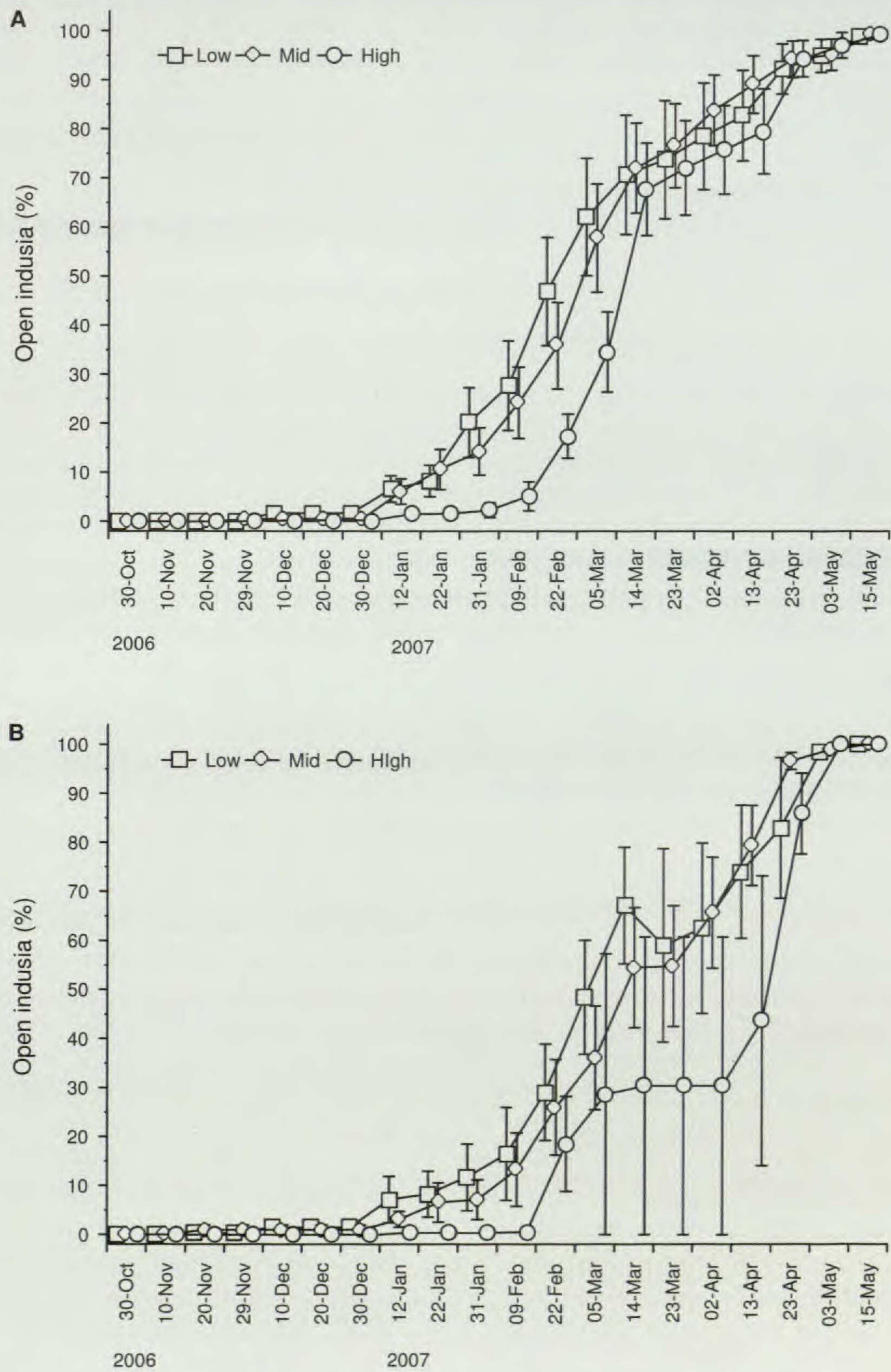
FIG. 4. Seasonal changes in opening of indusia (percent mean ± SE) for *Culcita macrocarpa* (A) and *Woodwardia radicans* (B) at three altitudes. Each mean represents twelve permanently marked individuals (n = 12). One fertile pinnule per individual was observed.

Release took place earlier at low altitude [54 Julian days (= February 23) ± 8 days, data of both species pooled] than at high altitude [80 Julian days (= March 21) ± 5 days], whereas release date at mid altitude [72 Julian days (= March 13) ± 6 days]) was not significantly different from those at high and low altitudes ($P > 0.05$, LSMEANS). Release date showed a significant positive correlation with maturation date both in C. macrocarpa ($r_s = 0.530$, $P = 0.001$, n = 35 individuals, Spearman rank correlation) and W. radicans ($r_s = 0.504$, $P = 0.007$, n = 27 individuals).

DISCUSSION

Environmental variables.—As expected, we found a decrease in temperature with an increase in altitude. The average altitudinal temperature gradient ($-0.78°C/100$ m) at Serra da Tronqueira was higher than those of other similar latitude regions. For example, in the Iberian Peninsula the gradient is $-0.59°C/100$ m in the northwestern coast (Carballeira et al., 1983) and in the central mountain range (Wilson et al., 2005). We also found an altitudinal moisture gradient. As in other Azorean islands (Borges, 1999), humidity increased with increasing altitude. Canopy cover varied among the three study sites as a consequence of different forest managements. At low and mid altitudes, the cover of the laurel forests was 71% and 63%, respectively. These percentages are lower than laurel forests in the Canary islands, with >90% canopy cover (Delgado et al., 2007). At the low altitude site, the forest was highly disturbed, with a young native tree canopy invaded by P. undulatum and A. melanoxylon. At mid altitude, the management has opened gaps in the tree canopy where exotic species (mainly C. arborea) have been removed (SPEA, 2007). At high altitude, there was a dense mature C. japonica plantation creating a canopy cover of 83%, the highest of all the sites.

Spore maturation and abortion.—We determined the timing of spore maturation on the basis of perispore formation and lipid-drops accumulation. The spores of C. macrocarpa and W. radicans matured by autumn (mean maturation date November 11 and December 11, respectively). In both species spores have a high lipid and caloric content (Arosa et al., 2009), as in ferns in general (Hew and Wong, 1974), but both spore mother cells and spores lack chloroplasts. Consequently, spore production may be largely controlled by the accumulation of enough photosynthates, as suggested for fruit production (French, 1992). In flowering plants, fruiting peaks generally occur during periods of low photosynthetic activity or after periods of high rates of reserve accumulation (Jordano, 1992). In temperate regions, fruiting has a unimodal peak in late summer or autumn (Jordano, 1992). The studied species mature spores in these seasons, as do many other temperate ferns (e.g., Page, 1997; Sawamura et al., 2009), suggesting that production of seeds and spores are governed by analogous selective pressures. Leaf expansion of C. macrocarpa and W. radicans ends in early summer (Quintanilla, 2002). Thus autumn spore maturation depends on photosynthate accumulation during summer and autumn.

Maturation date was neither affected by altitude nor by canopy cover for either species. This indicates that temperature, humidity and light variation along the altitudinal gradient did not constrain resource build-up for spore production. Körner and Diemer (1987) found that the optimum temperature for photosynthesis of lowland herbaceous flowering plants was ~23°C in a temperate climate. In our study, temperatures were frequently close to this optimum in the three altitudes, especially during the summer. Relative humidity was also high in the three altitudes during the summer and autumn months (means > 85%) and thus water availability must not be a limiting factor. The photosynthetic responses to light have been studied in very few fern species (Page, 2002). *Hymenophyllum tunbrigense* and *H. wilsonii*, filmy-ferns that grow on *C. macrocarpa* shoots in the study area, show photosynthetic saturation at low light levels (Proctor, 2003). A similar ecophysiological response in the study species would explain why maturation date was not affected by the differences in canopy cover among sites (maximum 83%, minimum 63%).

Spore abortion can indicate environmental stress during sporogenesis. However, both species had few aborted spores at all three altitudes (means ≤ 8%) suggesting that environmental conditions are optimal for their growth. Values were similar to those obtained from *Dryopteris* spp. (< 10%) in populations in northern Spain (Quintanilla and Escudero, 2006).

Spore release.—In both species there was a long period between spore maturation (autumn) and release (late winter). Since spore dissemination is the only function of the sporangia, we might expect its phenology to be influenced by selective pressures which would favor successful dispersal. Delayed release could be a strategy to avoid unfavorable winter conditions. However, mean temperatures during winter were 9°C to 12°C depending on altitude (Fig. 1A), which are suitable for spore germination of the studied species (Quintanilla *et al.*, 2000).

The long-term causes of the timing of spore maturation and release can also be biotic pressures such as seasonal presence of spore feeders. Some temperate ferns are attacked by a variety of spore-predator insects which occasionally cause severe spore reduction (Sawamura *et al.*, 2009, and references therein). We have not observed spore-feeding insects in the study species but consumption by the Azores bullfinch is significant (Ramos, 1995; Arosa *et al.*, in press). Given that Azores bullfinch consumes mature spores, we have studied its potential disperser role and the results will be reported elsewhere. In short, many droppings contained high amounts of viable (able to germinate) spores of *C. macrocarpa* and *W. radicans* and thus may provide a vehicle for dispersal. The pattern of autumn spore maturation and late winter release occurs throughout the range of both ferns (own observation), while the Azores bullfinch is present only in a small fraction (one island). Thus, the negative (predation) or positive (dispersal) interaction with the Azores bullfinch may not be important for determining the timing of spore dispersal.

Spore dispersal may be largely influenced by evolutionary constraints. Related plants share similar inherent design constraints which would limit

their potential evolutionary response to selection (Fenner, 1998). In ferns, both indusia and sporangia openings are passively caused by evaporative forcing. This must be an adaptation to favor long-distance wind dispersal of spores in warm dry days. *Culcita macrocarpa* and *W. radicans* released spores a month earlier at low altitude than at high altitude. This is due to the humidity and temperature gradients (see above) that reduce the evaporative forcing at high altitude (Körner, 2007). Delayed spore release may be merely due to the absence of dry weather conditions during most autumn and winter days. Release date was positively correlated with maturation date, i.e., individuals with earlier spore maturation showed earlier spore release, indicating that there is some interdependence between these events.

Implications for conservation of Azores bullfinch.—*Culcita macrocarpa* and *W. radicans* spores, together with seeds of the exotic *C. arborea*, are the main winter foods for the Azores bullfinch (Ramos, 1995; 1996a). The birds took the whole sorus, rejecting the indusium. After spore release, sori are not consumed since empty sporangia have negligible nutritional value. Food supply is at its lowest at the end of winter and the mortality of first-year birds appeared greater in this period (Ramos, 1995, 1996b). A gradual release of spores along the altitudinal gradient is important for the maintenance of a stock of spores throughout the winter. We can envisage that the Azores bullfinch distribution should be progressively pushed up to higher altitudes along the winter season following spore availability. For effective conservation of Azores bullfinches, the populations of *C. marcrocarpa* and *W. radicans* must be increased along a wide altitudinal range. This is significant in terms of habitat restoration for the Azores bullfinch because present management actions aim to control the expansion of *C. arborea* (Ceia, 2008). This invasive tree, although important in the winter diet, has a negative effect in spring because it outcompetes the native *I. perado* and *P. lusitanica* (the flower buds of both species are the main early spring foods for the Azores bullfinch; Ramos, 1996b).

Conclusions.—*Culcita macrocarpa* and *W. radicans* have similar timing of spore maturation and release. Maturation is completed in autumn and is not affected by altitude nor by canopy cover. Spore production may be largely controlled by the previous accumulation of photosynthates. Spores of both species are not released until late winter due to a requirement for dry weather conditions. Dispersal occurs earlier at lower altitude, as a consequence of higher temperature and lower humidity. The present study is descriptive and thus cannot accurately establish cause-effect relationships. Transplant experiments moving individuals from one habitat to another or experiments manipulating the physical environment experienced by individual ferns will clarify the relative importance of environmental and genetic factors on spore phenology traits.

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